THE SUBZERO TEMPERATURE STABILIZED OXYFERRO COMPLEX OF PURIFIED CYTOCHROME $P450_{scc}$

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1. Introduction

Purified preparations of bovine adrenocortical mitochondrial cytochrome $P450_{scc}$ capable of catalyzing the side-chain cleavage of cholesterol have been obtained [1,2]. The spectral, biochemical and immunological characteristics of this hemoprotein are now known [3,4], and the multistep nature of this bioconversion leading through enzyme-bound intermediates to pregnenolone formation, established [5–7].

The first step in the activation of molecular oxygen by cytochrome P450 involves the formation of an oxyferro complex [8]. Although oxyferro intermediates of a number of cytochrome P450s have been described [9] no information is available about this form of cytochrome $P450_{scc}$. The development of subzero temperature techniques [10–13] together with our studies on proton activity, dielectric constants and viscosity in fluid hydroorganic media, enabled the stabilization and detection of intermediates in various biological reactions [14–16]. Among these are the oxyferro species of cytochrome $P450_{cam}$ from *Pseudomonas putida* [17] and the highly purified microsomal cytochrome P450 (LM) from rabbit liver [18]. Here we describe the formation and stabilization at low temperature of the oxyferro intermediate of cytochrome $P450_{scc}$ isolated from bovine adrenocortical mitochondria.

2. Materials and methods

Cytochrome $P450_{scc}$ was prepared by the method in [2] and stored at −80°C in 50 mM potassium phosphate buffer pH 7.4. The content of endogeneous cholesterol was estimated by gas chromatography on a Girdel model 300 instrument equipped with an OV-17 column, using 4-cholesten-3-one as an internal standard [7]. In addition to cholesterol (2.75 mol/mol $P450_{scc}$) we demonstrated the presence of small amounts (0.1–0.4 mol/mol $P450_{scc}$) of the oxygenated sterol intermediates, (22*R*)-22-hydroxycholesterol and (20*R*,22*R*)-20,22-dihydroxycholesterol, as natural constituents of the substrate-bound form of cytochrome $P450_{scc}$, which may contribute to the mixed spin state (55% low spin) of the preparation [7,19]. The purity of the protein was determined by the A_{414}/A_{280}. In the glycerol-induced low spin configuration our cytochrome $P450_{scc}$ (Fe^{3+}_{S}) preparation gave a ratio of 0.65. SDS electrophoresis confirmed that the apoprotein moved as a single band.

Spectra were recorded on an Aminco-Chance DW2 spectrophotometer equipped with a low temperature thermoregulatory device [14]. Mixed solvents for spectral analysis, consisting of equal volumes of 0.1 M Tris–acetate buffer (pH 7.5) and glycerol (GlOH), were thoroughly deoxygenated by sonication in vacuo followed by purging with purified Argon for 15 min. After addition of cytochrome $P450_{scc}$ and a photoreducing system, deoxygenation was continued at 4°C for an extra 1 h by passing a gentle stream of purified argon over the surface. The photochemical reduction system consists of 1 mM EDTA and 10 μM each of acridine orange and methyl viologen [20]. All solvents and chemicals were of the highest purity commercially available and used without further purification.

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3. Results and discussion

3.1. *Formation of the oxyferro–cytochrome P450$_{scc}$ complex*

In the presence of 50% GlOH the Fe^{3+}_S cytochrome P450$_{scc}$ preparation (2.3 μM) was entirely in the low spin configuration [21]. After deoxygenation of the reaction mixture was complete the cuvette was maintained at 4°C and the ferric heme was photoreduced using white light. After 50 min no further spectral changes occurred indicating that the reduction was completed. The sample cuvette was rapidly cooled to −30°C and the oxyferro complex was formed by gently purging with oxygen at 30–50 ml/min for 10 s. During these procedures no significant spectral or thermal perturbations of the sample occurred.

3.2. *Spectral characterisation of the oxyferro intermediate*

Under the above experimental conditions, we could begin spectroscopic observations within 5 s of the addition of O_2. A new species, with an absorption spectrum distinctly different from that of the stable ferric or ferrous form of cytochrome P450$_{scc}$ is obtained (fig.1). Its spectrum, which is characterized by A_{max} at 422 and 555 nm, remains stable for several hours at −30°C in water/GlOH, 1:1 (v/v). The spectrum of this new species is very similar to that of other $Fe^{2+}_S \cdot O_2$ complexes prepared under similar conditions [17,18]. Table 1 summarizes principal ϵ values for different forms of cytochrome P450$_{scc}$ under various experimental conditions. The ϵ values were calculated assuming a $\Delta\epsilon_{448} - \Delta\epsilon_{490} = 91\ mM^{-1}.cm^{-1}$ for the difference spectrum of $Fe^{2+}_S \cdot CO - Fe^{2+}_S$.

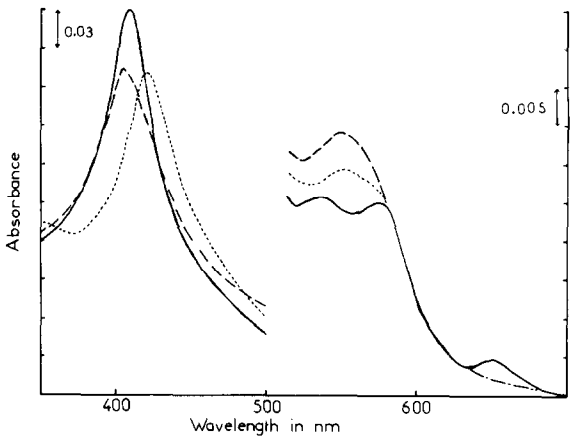

Fig.1. Absolute spectra of 2.3 μM solubilized adrenocortical cytochrome P450$_{scc}$ in 0.1 M Tris buffer (pH 7.5)/GlOH, 1:1 (v/v). (——) Oxidized form of cytochrome P450$_{scc}$ at +4°C; (– – –) reduced form of cytochrome P450$_{scc}$ at −30°C; (- - - -) idem after oxygenation at −30°C.

At <−15°C the oxyferro intermediate of cytochrome P450$_{scc}$ remains stable for ≥3 h. At −15°C a first transition is observed, the maximum in the Soret region progressively shifts to 412 nm (fig.2). This new spectrum also remains stable for several hours. Towards 0°C decomposition occurs and the spectrum returns to that of the original Fe^{3+}_S complex. The two spectra obtained at subzero temperature may represent two different forms of the oxyferro complex of P450$_{scc}$, this has been suggested for the similar phenomena observed with microsomal P450 [9]. Clarification of these spectral changes at −15°C await further experimentation.

Our observations confirm the existence of a cyto-

Table 1
Extinction coefficients (mM . cm^{-1}) of various forms of cytochrome P450$_{scc}$

Compounds	Wavelength (nm)						
	393	412	414	422	552	556	570
Fe^{3+} (LS)[a]			121				
Fe^{3+} (HS)[a]	103						
$Fe^{3+} \cdot S$[b]			120 ± 1				11 ± 1
$Fe^{2+} \cdot S$[b]		87 ± 1			14 ± 1		
$Fe^{2+} \cdot S$[c]		88 ± 1			15 ± 1		
$Fe^{2+} \cdot S{-}O_2$[c]			84 ± 2			12 ± 1	

[a] From [22];
[b] In GlOH–water buffer 1:1 (v/v) at +5°C;
[c] In GlOH–water buffer 1:1 (v/v) at −30°C

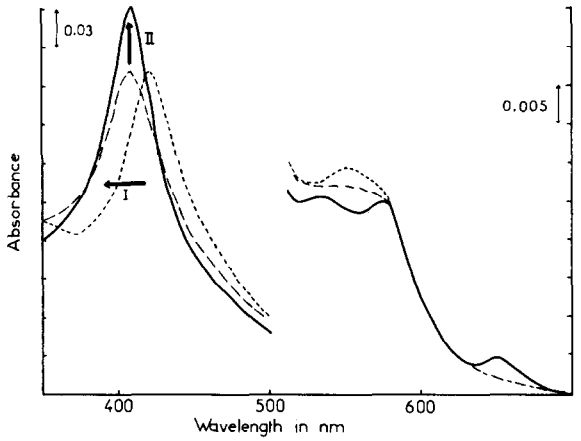

Fig.2.Decomposition of the oxyferro–cytochrome $P450_{scc}$ complex prepared at −30°C: I, First step occurring at −15°C; II, second step occurring towards 0°C.

chrome $P450_{scc}$–$Fe^{2+}_{S} \cdot O_2$ species with spectral properties which closely resemble those of other cytochrome P450 oxygen complexes. This finding supports the idea that all P450-dependent oxygen activation processes involve a similar $Fe^{2+} \cdot O_2$ intermediate. Furthermore this suggests that the 3 distinctly different activities exhibited by this enzyme, e.g., 22- and 20-hydroxylase and 20,22-desmolase, also involve 3 consecutive but identical oxygen activation cycles of the heme iron [6,7]. The stabilization of the cytochrome $P450_{scc}$–oxyferro complex at subzero temperatures as an early step of these oxidation cycles predicts that this technique (low temperature spectrophotometry) has great potential for visualising subsequent species in the oxygen activation process. Studies to this end, using hydroperoxysterols as a combined substrate/oxygen-donor model, are in progress.

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